



Patent Application No. : 10/568,300  
Examiner of USPTO : SALMON, Katherine D  
Title of the Invention : METHOD FOR THE DETECTION OF CYTOSINE  
METHYLATIONS IN DNA WITH THE AID OF SCORPION  
Applicant : Epigenomics AG  
Filing Date : August 08, 2003

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**DECLARATION OF Dr. JÜRGEN DISTLER IN THE NAME OF APPLICANT**  
**EPIGENOMICS AG**

I, Jürgen Distler, declare and state as follows:

1. I am currently **Vice President Product Development** at Epigenomics AG, co-applicant of the above-identified patent application (the "Application").
2. My scientific Curriculum Vitae, including a list of my publications, is attached to and forms part of this Declaration.
3. I am familiar with the specification and claims of patent application No. 10/568,300 and the prior art cited in the proceedings before the U.S. Patent Office.
4. As evident from the enclosed CV, I have been involved in biochemical research since I finished my studies in biology at the University of Munich in 1984.
5. I joined Epigenomics AG in 1999, and in the field of DNA methylation I have been involved in as part of my responsibilities:
  - Method Development (Research)
  - Assay Format Development (Research)
  - Product Development
  - Manufacturing of Products (CE-certified)
6. Several publications in peer-reviewed scientific journals verify my expertise in the field of DNA methylation. I am also an inventor on a number of patents and patent applications (see patents and applications listed in my CV).

7. It is fair to say that I by the time have profound knowledge not only of bisulfite-based methods and the DNA methylation-based diagnostic technology described in the application, but also of the general knowledge available in the field by the time the application was filed.
8. I submit this declaration as evidence of the non-obviousness of the presently claimed method that is based on a bisulfite reaction, combined with the application of methylation specific Scorpion primers, over the prior art references cited in the Office Actions issued during the examination proceedings of the application. As explained herein above, I am familiar with the application and have reviewed the final Office Action dated March 12, 2010, in addition to the documents referred to by the Examiner in this Office Action.
9. I provide my opinion on the invention as defined in the claims currently pending, taking into account the disclosure of the application as originally filed, the general knowledge available in the art at the time the application was filed, and the teachings from the below referenced prior art.
10. The present invention relates to an improved method for the detection of cytosine methylation in DNA that is based on a bisulfite reaction and the subsequent application of methylation specific Scorpion primers. The presently claimed method is useful for determining methylation positions in a nucleic acid which, in turn, is of interest for diagnostic purposes in the field of epigenomics.
11. At the time of filing the present application in 2003, methods comprising the use of Scorpion primers, i.e. primers whose 5'-ends are joined with a probe via a linker and wherein the probe subsequently hybridizes intramolecularly to the primer extension product, were limited to the detection of mutational alterations in DNA. The use of such primers, however, for the detection of initially methylated cytosines within bisulfite treated DNA, was not known in 2003.
12. At the time of filing the present application, it was unknown to the person of ordinary skill in the art that Scorpion primers are suitable when applied for the detection of cytosine methylation in methods directed to the analysis of bisulfite treated DNA. Moreover, the skilled person would have expected erroneous false-positive or false-negative signalling to occur when using Scorpion primers with methylation-specific probe sequences. This is due to the functional principle of Scorpion primers as described below.

13. At the 5' end, a Scorpion primer carries a self-complementary stem sequence, the function of which is to keep two molecules, a fluorescent dye and a quencher in spatial proximity to each other. Therefore, prior to the primer extension reaction, no signal is emitted (alternative variants exist; like FRET, where a signal is emitted if both molecules are in spatial proximity). After hybridization to the target DNA, the primer is extended by a polymerase. Subsequently, the self-complementary stem sequence is denatured and the probe sequence hybridizes to a part of the primer extension product. This results in the separation of the fluorescent dye from the quencher and causes the emission of a signal.
14. To ensure a stable hybridization, the self-complementary stem sequence mainly consists of G and C nucleotides (Thelwell, et al. Nucleic Acids Research, Vol. 28,19, p. 3752-3761, 2000). However, the detection of initially methylated cytosine positions by hybridizing probe sequences requires the probes to include CG dinucleotides as well, whereas the specificity of the probe increases with the number of CG positions it contains. Therefore, internal base pairings between the stem sequence and a methylation-specific probe sequence are likely to occur. By the time of filing the present application, the person skilled in the art was aware of this significant risk of internal base pairings. The skilled person also was unable to predict the internal base pairing behaviour between a GC rich methylation specific probe and a GC rich sequence located in the stem structure of the primer during the polymerase reaction cycles.
15. Regarding the present invention, it was therefore surprising that the phenomenon of internal hybridization did not have the expected impact on the results and that Scorpion primers with methylation-specific probe sequences could be successfully applied for methylation analysis.
16. I declare that all statements made herein are, to my own knowledge, true and that all statements made on information and belief are believed to be true. I also declare that these statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, and that such wilful false statements may jeopardize the validity of the captioned patent application or any patent issued therefrom.

Date September 09, 2010

By

  
Dr. Jürgen Distler

Encl

Curriculum Vitae

List of Scientific Publications

List of Presentations at Scientific Meetings

List of selected Patents / Patent Applications



## **Curriculum Vitae**

**Dr. Jürgen Distler**  
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### **I. Personal Data:**

Date of Birth: September 11, 1958  
Place of Birth: Kempten/Allgäu, Germany

### **II. Academic Background:**

Dipl.-Biologist  
University of Munich  
1984

1984-1988: PhD Technical University Darmstadt  
1988-1989: Postdoctoral Fellow Institute Pasteur, Paris  
1990- 1998: Scientific Assistant University of Wuppertal

### **III. Employment History and Experience**

2008 – present:	Vice President Product Development Epigenomics AG Kleine Präsidentenstr. 1 10178 Berlin Germany
2005 – 2008:	Senior Manager Product Development Epigenomics AG Kleine Präsidentenstr. 1 10178 Berlin
2003 – 2004:	Project Manager Assay Technologies Epigenomics AG Kleine Präsidentenstr. 1 10178 Berlin
1999 – 2002:	Scientist Research Epigenomics AG Kleine Präsidentenstr. 1 10178 Berlin
1998- 1999:	Scientific Consultant for several Biotec Companies

#### IV. Scientific Publications

1: Tänzer M, Balluff B, Distler J, Hale K, Leodolter A, Röcken C, Molnar B, Schmid R, Lofton-Day C, Schuster T, Ebert MP. Performance of epigenetic markers SEPT9 and ALX4 in plasma for detection of colorectal precancerous lesions. *PLoS One*. 2010 Feb 4;5(2):e9061. PubMed PMID: 20140221; PubMed Central PMCID: PMC2816214.

2: Fassbender A, Lewin J, König T, Rujan T, Pelet C, Lesche R, Distler J, Schuster M. Quantitative DNA methylation profiling on a high-density oligonucleotide microarray. *Methods Mol Biol*. 2010;576:155-70. PubMed PMID: 19882262.

3: deVos T, Tetzner R, Model F, Weiss G, Schuster M, Distler J, Steiger KV, Grützmann R, Pilarsky C, Habermann JK, Fleshner PR, Oubre BM, Day R, Sledziewski AZ, Lofton-Day C. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. *Clin Chem*. 2009 Jul;55(7):1337-46. Epub 2009 Apr 30. PubMed PMID: 19406918.

4: Weiss G, Cottrell S, Distler J, Schatz P, Kristiansen G, Ittmann M, Haefliger C, Lesche R, Hartmann A, Corman J, Wheeler T. DNA methylation of the PITX2 gene promoter region is a strong independent prognostic marker of biochemical recurrence in patients with prostate cancer after radical prostatectomy. *J Urol*. 2009 Apr;181(4):1678-85. Epub 2009 Feb 23. PubMed PMID: 19233404.

5: Distler J. Quantification of methylated DNA by HeavyMethyl duplex PCR. *Methods Mol Biol*. 2009;507:339-46. PubMed PMID: 18987825.

6: Lofton-Day C, Model F, Devos T, Tetzner R, Distler J, Schuster M, Song X, Lesche R, Liebenberg V, Ebert M, Molnar B, Grützmann R, Pilarsky C, Sledziewski A. DNA methylation biomarkers for blood-based colorectal cancer screening. *Clin Chem*. 2008 Feb;54(2):414-23. Epub 2007 Dec 18. PubMed PMID: 18089654.

7: Tetzner R, Dietrich D, Distler J. Control of carry-over contamination for PCR-based DNA methylation quantification using bisulfite treated DNA. *Nucleic Acids Res*. 2007;35(1):e4. Epub 2006 Nov 28. PubMed PMID: 17135186; PubMed Central PMCID: PMC1747185.

8: Schatz P, Distler J, Berlin K, Schuster M. Novel method for high throughput DNA methylation marker evaluation using PNA-probe library hybridization and MALDI-TOF detection. *Nucleic Acids Res*. 2006 May 2;34(8):e59. PubMed PMID: 16670426; PubMed Central PMCID: PMC1456329.

9: Cottrell SE, Distler J, Goodman NS, Mooney SH, Kluth A, Olek A, Schwöpe I, Tetzner R, Ziebarth H, Berlin K. A real-time PCR assay for DNA-methylation using methylation-specific blockers. *Nucleic Acids Res*. 2004 Jan 13;32(1):e10. PubMed PMID: 14722226; PubMed Central PMCID: PMC373310.

10: Adorján P, Distler J, Lipscher E, Model F, Müller J, Pelet C, Braun A, Flori AR, Gütig D, Grabs G, Howe A, Kursar M, Lesche R, Leu E, Lewin A, Maier S, Müller V, Otto T, Scholz C, Schulz WA, Seifert HH, Schwöpe I, Ziebarth H, Berlin K, Piepenbrock C, Olek A. Tumour class prediction and discovery by microarray-based DNA methylation analysis. *Nucleic Acids Res.* 2002 Mar 1;30(5):e21. PubMed PMID: 11861926; PubMed Central PMCID: PMC101257.

11: Stratmann A, Mahmud T, Lee S, Distler J, Floss HG, Piepersberg W. The AcbC protein from *Actinoplanes* species is a C7-cyclitol synthase related to 3-dehydroquinate synthases and is involved in the biosynthesis of the alpha-glucosidase inhibitor acarbose. *J Biol Chem.* 1999 Apr 16;274(16):10889-96. PubMed PMID: 10196166.

12: Küster C, Piepersberg W, Distler J. Cloning and transcriptional analysis of the *rplKA*-or *f31-rplJL* gene cluster of *Streptomyces griseus*. *Mol Gen Genet.* 1998 Jan;257(2):219-29. PubMed PMID: 9491081.

13: Ahlert J, Distler J, Mansouri K, Piepersberg W. Identification of *stsC*, the gene encoding the L-glutamine:scyllo-inosose aminotransferase from streptomycin-producing *Streptomyces*. *Arch Microbiol.* 1997 Aug;168(2):102-13. PubMed PMID: 9238101.

14: Lyutskanova D, Distler J, Altenbuchner J. A spectinomycin resistance determinant from the spectinomycin producer *Streptomyces flavopersicus*. *Microbiology.* 1997 Jul;143 ( Pt 7):2135-43. PubMed PMID: 9245803.

15: Thamm S, Distler J. Properties of C-terminal truncated derivatives of the activator, StrR, of the streptomycin biosynthesis in *Streptomyces griseus*. *FEMS Microbiol Lett.* 1997 Apr 15;149(2):265-72. PubMed PMID: 9141668.

16: Beyer S, Distler J, Piepersberg W. The *str* gene cluster for the biosynthesis of 5'-hydroxystreptomycin in *Streptomyces glaucescens* GLA.0 (ETH 22794): new operons and evidence for pathway-specific regulation by StrR. *Mol Gen Genet.* 1996 Apr 10;250(6):775-84. PubMed PMID: 8628239.

17: Retzlaff L, Distler J. The regulator of streptomycin gene expression, StrR, of *Streptomyces griseus* is a DNA binding activator protein with multiple recognition sites. *Mol Microbiol.* 1995 Oct;18(1):151-62. PubMed PMID: 8596455.

18: Kuberski S, Kasberg T, Distler J. The *nusG* gene of *Streptomyces griseus*: cloning of the gene and analysis of the A-factor binding properties of the gene product. *FEMS Microbiol Lett.* 1994 Jun 1;119(1-2):33-9. PubMed PMID: 8039667.

19: Distler J, Mansouri K, Mayer G, Stockmann M, Piepersberg W. Streptomycin biosynthesis and its regulation in *Streptomyces*. *Gene.* 1992 Jun 15;115(1-2):105-11. PubMed PMID: 1377151.

20: Heinzel P, Werbitzky O, Distler J, Piepersberg W. A second streptomycin resistance gene from *Streptomyces griseus* codes for streptomycin-3"-phosphotransferase. Relationships between antibiotic and protein kinases. Arch Microbiol. 1988;150(2):184-92. PubMed PMID: 2844130.

21: Distler J, Ebert A, Mansouri K, Pissowotzki K, Stockmann M, Piepersberg W. Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: nucleotide sequence of three genes and analysis of transcriptional activity. Nucleic Acids Res. 1987 Oct 12;15(19):8041-56. PubMed PMID: 3118332; PubMed Central PMCID: PMC306325.

23: Distler J, Braun C, Ebert A, Piepersberg W. Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: analysis of a central region including the major resistance gene. Mol Gen Genet. 1987 Jun;208(1-2):204-10. PubMed PMID: 3039306.

## V. Presentations at Scientific Meetings

92<sup>nd</sup> Annual Conference of the German Pathology Society e.V.  
- Week of Pathology - Berlin, Germany (2008)

Poster: "Early detection of prostate cancer using DNA methylation analysis of the GSTP1 gene in histopathologically negative prostate cancer specimens: potential to improve the clinical routine?"

CNAPS-V Conference, Moscow, Russia (2007)

Presentation: "Septin 9 Methylation as a Plasma Biomarker for Colorectal Cancer"

Distler, J. (1991) Streptomycin biosynthesis and its regulation in *Streptomyces griseus*. International Symposium on the Biology of Actinomycetes. ISBA '91; University of Wisconsin-Madison, USA.

Distler, J. (1996) Linking primary and secondary metabolism in *Streptomyces griseus*. European Conference on Biology of Streptomycetes, Ohrbeck.

Distler, J. (1996) Verbreitung und Entstehung von Antibiotika-Resistenzmechanismen in Antibiotika-Produzenten. „Wirkungs- und Resistenzmechanismen von Antibiotika“; Medizinische Hochschule Hannover.

Distler, J. (1997) Regulation of streptomycin biosynthesis in *Streptomyces griseus*- linking primary and secondary metabolism. International Symposium on Biology of Actinomycetes, ISBA '97; Beijing, PR of China.

Distler, J. (1997) Streptomycin biosynthesis in *Streptomyces griseus* - a model analysing the regulatory network inducing secondary metabolism. Japan-UK *Streptomyces* Workshop, John Innes Inst., Norwich, UK.

Distler, J. (1998) Antibiotikasyntese in Actinomyceten. Biochemie. Genetik und Regulation. Biochemisches Seminar; Universität Münster

Distler, J. (1998) Regulation of the biosynthesis of streptomycin and related antibiotics. 8th International Symposium on the Genetics of Industrial Microorganisms; Jerusalem, Israel.

Distler, J. (1998) Regulation and physiology of streptomycin biosynthesis in *Streptomyces griseus*. Portrait of an organism: the genetic analysis of *Streptomyces coelicolor* A3(2) biology- A symposium to mark the retirement of Sir David Hopwood FRS. Organised by the Society for General Microbiology and the Genetical Society; Norwich, UK.

Distler, J. (1999) Biological diversity from microbial sources in combinatorial biochemistry. 6<sup>th</sup> Conference on the Biotechnology of Microbial Products (BMP '99); San Diego, USA.

## VI. Selected Patents and Patent Applications

Piepersberg, W., Stockman, W., Distler, J., Mansouri, K., Grabley, S., Sichel, P., Bräu, B. (1991) "Verfahren zur Isolierung von Sekundärmetabolit-Biosynthesegene aus Actinomyceten sowie deren Verwendung", Deutsche Patentanmeldung P41 30 967- HOE 91/F 300.

Piepersberg, W., Distler, J., Stratmann, A., Crueger, A. (1995) Acarbose-Biosynthesegene aus *Actinoplanes sp.*, Verfahren zu ihrer Isolierung sowie ihrer Verwendung. Deutsche Patentanmeldung (Le A 30 515), (also international submission).

Marquat, R., Hoersch, B., Seiffert-Störko, A., Stein, A., Zervosen, A., Elling, L., Kula, M.-R., Verseck, S., Distler, J., Piepersberg, W. (1996) Process for the enzymatic synthesis of nucleotide-6-deoxy-d-xylo-4-hexuloses, Europäische Patentanmeldung, 96115222.0-2116 United States Letters Patent, 08/731,189.

Crueger, A., Dellweg, H.-G., Lenz, J.-G., Schröder, W., Pape, H., Goeke, K., Schaper, B., Hemker, M., Piepersberg, W., Distler, J., Stratmann, A. (1997) Verfahren zur Herstellung sowie der Verwendung von Acarviosyl-Transferasen bei der Umwandlung von Acarbose-Homologen in Acarbose, zur Herstellung von Acarbose-Homologen. Europäische Patentanmeldung, 97104115.7-2105.

Piepersberg, W., Albermann, C., Distler, J. (1997) Verfahren zur enzymatischen Synthese von GDP-6-Deoxyhexosen und deren Verwendung zur Herstellung von Oligosacchariden. Deutsche Patentanmeldung.